

Remarks


In response to the Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosures, mailed on June 14, 2001, Applicants submit herewith a paper copy and a computer readable copy of a substitute Sequence Listing and accompanying statement. Although the Notice did not require a paper copy of the Sequence Listing, Applicants are submitting a paper copy herewith, since the originally filed Sequence Listing did not recite all the sequences disclosed in the application. The substitute Sequence Listing is believe to list all the sequences disclosed in the application. No new matter has been added.

This amendment also corrects inadvertent typographical errors in the specification and in Fig.8 and amends claims 26 and 45. Support for the claim amendment can be found, e.g., at page 22, lines 1 to 9 of the specification. No new matter has been added.

Conclusion

No fees are believed to be due with this Response and Amendment. If, however, any fees are due, the Commissioner is hereby authorized to charge the necessary fees to our Deposit Account No. 06-1448.

Respectfully submitted,

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Copy of replacement paragraphs with changes marked thereon

Replacement paragraph bridging pages 3 and 4:

In yet another preferred embodiment, the host cell is further modified to increase its ability to proliferate. The modification can, e.g., increase the reducing capacity of the cytoplasm sufficiently to increase the growth of the host cell. The modification can be a mutation in a gene, e.g., a suppressor mutation, or it can an introduction and expression of a gene encoding a growth promoting protein into the host cell. In a preferred embodiment, the gene encoding the AphC subunit of the alkyl hydroperoxidase is mutated in the host cell, e.g., by the presence of a mutation in the TCT triplet rich region of the gene (see Figure 8A). In another embodiment, a gene encoding a mutated form of AphC is introduced and expressed in the host cell. Such host cells preferably have a growth curve that is similar to that of the wild type parent strain. Particularly preferred host cells are the host cells described in the Examples, referred to as FA112 and FA113, which are *trxB* [*gsha*] *gshA* *supp* and *trxB* *gor* *supp* mutants, respectively. These two strains have been deposited at the American Type Culture Collection (ATCC) 10801 University Blvd., Manassas, VA 20110-2209 on November 11, 1999, in accordance with the terms and provisions of the Budapest Treaty relating to the deposit of microorganism. FA112 and FA113 have been assigned ATCC Accession No. PTA-938 and PTA-939, respectively.

Replacement paragraph for page 4, lines 4-9:

The host cell can comprise a nucleic acid encoding a catalyst of disulfide bond[r] bond isomerization, e.g., variants of a thioredoxin or glutaredoxin, which have, e.g., a redox potential that is higher than that of its wild type counterpart. In an illustrative example, the variant is a "Grx" variant of thioredoxin A. The host cell can also comprise a catalyst of disulfide bond isomerization, such as a disulfide bond isomerase, e.g., DsbC, or derivative thereof.

Replacement paragraphs for page 5, lines 32-38:

Figure 8B is an alignment of amino acid sequences of AhpC proteins from different microorganisms and from the human species (HUMAN_[TPA] TSA). The numbers represent the amino acid position of the first amino acid shown in each protein. The sequences correspond, from top to bottom, to SEQ ID Nos: 12-20.

Figure 9 is a [diagram] diagram showing the two different forms of AhpC that can be found in a cell depending on the oxidative stress-inducing signal. The form on the left represents the [wild-type] wild-type enzyme and the form on the right, the mutant enzyme.

Replacement paragraph for page 6, lines 20-40:

Host cells or organisms of the invention for the efficient production of disulfide bond containing proteins can be produced by various modifications or combinations of modifications of wild type cells or organisms or cells or organisms which have already been modified. In one embodiment, a host cell is modified by reducing or eliminating the level or activity of one or more [reductase] reductases in the host cell. In a preferred embodiment, the reductase is selected from the group consisting of the thioredoxin reductase (trxB); glutathione (gshA and gshB); and the glutathione oxidoreductase (gor). Such a host cell can further be modified to increase its rate of growth, if necessary, such as selecting naturally occurring mutants, e.g., suppressor mutants, or by the introduction of a mutation or a heterologous DNA or stimulating the expression or activity of a gene, thereby resulting in an increased growth rate of the host cell. A modification of a host cell resulting in improved growth is referred to herein as "growth inducing modification." Growth of modified host cells can be improved or restored to that of wild type host cells by increasing the reducing environment of the cytoplasm, preferably without affecting the oxidative environment necessary for appropriate oxidation of disulfide bond containing proteins. Accordingly, the oxidizing role of the thioredoxins in the host cell is preferably not modified. In one embodiment, a modified host cell is modified by altering the activity of the AphC subunit of the alkyl hydroperoxidase AhpCF, such as by mutating the region of the aphC gene containing four TCT triplets, so that the enzyme has a new reducing activity. A preferred *E. coli* bacterial strain having a mutated aphC gene is the strain FA113 which has been deposited at the ATCC and has been assigned ATCC Accession No. PTA-939.

Replacement paragraph for page 10, lines 34-39:

The term "disulfide bond isomerization" refers to an exchange of disulfide bonds between different cysteines, i.e., the shuffling of disulfide bonds (see Figure 1). Isomerization of disulfide bonds is mediated by thiol-disulfide exchange between the active site cysteines of enzymes and cysteines in the target protein (see Figure 1) and catalyzed by [isomeras] isomerases. In *E. coli*, isomerization is catalyzed by DsbC, a periplasmic disulfide bond oxidoreductase.

Replacement paragraph for page 11, lines 1-8:

"Protein disulfide bond isomerases" refer to proteins which catalyze the isomerization of disulfide bonds in proteins. Without wanting to be limited to a specific mechanism of action, isomerases are thought to act initially by invading incorrect disulfide bonds that have been formed in proteins and then allowing or promoting isomerization of the disulfide bond. To carry out this process, it is posited that the two cysteines in [teh] the Cys-Xaa-Xaa-Cys motif must be in the reduced state (Figure 1). In fact, DsbC is found with its [inactive] active site cysteines in the reduced state in wild-type *E. coli*. DsbC is maintained in a reduced state in a cell by the cytoplasmic membrane protein DsbD (or DipZ protein).

Replacement paragraph for page 11, lines 18-26:

The term "thioredoxin fold" refers to an overall protein structural motif that is shared by the members of the thioredoxin superfamily. Thus, although thioredoxins and glutaredoxins may have relatively different amino acid sequences, they share a similar secondary structure, i.e., a similar overall fold, referred to as the thioredoxin fold. The thioredoxin fold consists of a central four-stranded beta-sheet flanked by three alpha-helices [in the order] (see, e.g., Figure 1 in Jordan et al. (1997), *J. Bio. Chem.* 272:18044). The thioredoxin fold has been found in five distinct classes of proteins that have the common property of interacting with cysteine-containing substrates (see, e.g., Martin J.L.(1995) *Structure* 3: 245 and Aslund et al. (1996) *J. Biol. Chem.* 271:6736).

Replacement paragraph for page 12, lines 23-25:

"DsbB," which is encoded by the gene *dsbB*, is a cytoplasmic [] membrane protein which oxidizes DsbA. DsbB contains a Cys-Xaa-Xaa-Cys (SEQ ID NO: 1) (Xaa being any amino acid residue) motif. DsbB may be oxidized by passing electrons to the res[t]piratory respiratory chain.

Replacement paragraph bridging pages 15 and 16:

Suitable bacteria for this purpose include archaeobacteria and eubacteria, especially *eubacteria*, and most preferably *Enterobacteriaceae*. Other examples of useful bacteria include *Escherichia*, *Enterobacter*, *Azotobacter*, *Erwinia*, *Bacillus*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Rhizobia*, *Vitreoscilla*, and *Paracoccus*. Suitable *E. coli* hosts include *E. coli* DHB4, *E. coli* BL-21 (which are deficient in both [lon] Lon (Phillips et al. (1984) *J. Bacteriol.* 159: 283.) and [ompT] OmpT proteases), *E. coli* AD494, *E. coli* W3110 (ATCC 27,325), *E. coli* 294 (ATCC 31,446), *E. coli* B, and *E. coli* X1776 (ATCC 31,537). Other strains include *E. coli* B834 which are methionine deficient and, therefore, enables high specific activity labeling of target proteins with ³⁵S-methionine or selenomethionine (Leahy et al. (1992) *Science* 258, 987). Yet other strains of interest include the BLR strain, and the K-12 strains HMS174 and NovaBlue, which are *recA*- derivative that improve plasmid monomer yields and may help stabilize target plasmids containing repetitive sequences (these strains can be obtained from Novagen).

Replacement paragraph page 34, lines 26-39:

In an illustrative embodiment, disulfide bond containing proteins of the invention are produced as follows. A host cell or organism of the invention is first transformed with an expression plasmid encoding a polypeptide of interest and a selection marker. The plasmid can encode additional polypeptides, such as is desired, e.g., in the production of multi-polypeptide proteins. Additional plasmids encoding other polypeptides can be co-transformed, or transformed separately into the host cell or [organism] organism. When using more than one plasmid, it may be preferable to use different markers of selection, to insure that all the desired plasmids are contained

in the recombinant host cell that is selected. Following transformation of the one or more plasmids into the host cells, according to known methods, clones having taken up the plasmid(s) are selected on appropriate medium, and cloned. Separate clones are then tested to confirm that they have the desired characteristics, including the expression of the one or more polypeptides. In particular, the polypeptide(s) of interest can be isolated from the host cells, and tested for activity, amount, etc. The isolated clones can then be frozen in aliquots for preservation, pursuant to methods well known in the art.

Replacement paragraph bridging pages 38-39:

A protein of the invention be used in one or more of the following purposes or effects: inhibiting the growth, infection or function of, or killing, infectious agents, including, without limitation, bacteria, viruses, fungi and other parasites; effecting (suppressing or enhancing) bodily characteristics, including, without limitation, height, weight, hair color, eye color, skin, fat to lean ratio or other tissue pigmentation, or organ or bodypart size or shape (such as, for example, breast augmentation or diminution, change in bone form or shape); effecting biorhythms or [caricadic] circadian cycles or rhythms; effecting the fertility of male or female subjects; effecting the metabolism, catabolism, anabolism, processing, utilization, storage or elimination of dietary fat, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional factors or component(s); effecting behavioral characteristics, including, without limitation, appetite, libido, stress, cognition (including cognitive disorders), depression (including depressive disorders) and violent behaviors; providing analgesic effects or other pain reducing effects; promoting differentiation and growth of embryonic stem cells in lineages other than hematopoietic lineages; hormonal or endocrine activity; in the case of enzymes, correcting deficiencies of the enzyme and treating deficiency-related diseases; treatment of hyperproliferative disorders (such as, for example, psoriasis); immunoglobulin-like activity (such as, for example, the ability to bind antigens or complement); and the ability to act as an antigen in vaccine composition to raise an immune response against such protein or another material or entity which is cross-reactive with such protein.

Replacement paragraph for page 43, lines 1-7:

For establishing the growth curve of the FA113 strain and compare it to its wild type parent strain, the bacteria were subjected to aerobic growth at 37°C in LB medium in test tubes. The results, which are presented in Figure [3] 4, show that at 37°C in rich media, FA113 was found to grow almost as well as the wild type (DHB4, *trxB⁺ gor⁺*) strain with doubling times 30 and 27 minutes, respectively. In contrast, WP778, the *trxB gor* parent of FA113 grew with a doubling time of 300 min in the absence of DTT (Prinz, et al. (1997) *J. Biol. Chem.* 272: 15661).

Replacement paragraph for page 46, lines 10-11:

Thus, properly formed and oxidized alkaline phosphatase forms in the cytoplasm of the [TrxB] trxB gor supp mutant FA113.

Replacement paragraph for page 46, lines 15-28:

Stewart et al. (Stewart, et al. (1998) *EMBO J.* 17: 5543) have shown that disruption of trxB results in an accumulation of oxidized thioredoxins which can then act as oxidases, the reverse of their normal role. Likewise, in FA113, TrxA expressed from the chromosome was present solely in the oxidized form. We examined the effect of high level expression of TrxA and TrxA mutant proteins with varying redox potentials on the folding of the more complex multi-disulfide proteins, namely vtPA and tPA. The redox potential of most cysteine oxidoreductases, including TrxA, is strongly influenced by the sequence of the dipeptide within the CXXC (SEQ ID NO: 1) active site motif (Mossner, et al. (1999) *J. Biol. Chem.* 274: 25254; Mossner, et al. (1998) *Protein Sci.* 7: 1233; Grauschopf, et al. (1995) *Cell* 83: 947). TrxA with a wild type active site (-CGPC-; SEQ ID NO: 2) and five mutants with varying redox potentials (see below) were cloned into plasmid pBAD33 ([Gunzman] Guzman et al. (1995) *J. Bacteriol.* 177:4121) under the control of the araBAD promoter and transformed into FA113 together with a compatible expression vector for vtPA or full length tPA synthesis.

Replacement paragraph for page 47, lines 27-30:

Thus, the results of this Example show that cotransformation of TrxB gor supp mutant with a plasmid encoding a thioredoxin variant having a higher redox potential than its wild type [coutnerpart] counterpart significantly increases the production of proteins containing multiple disulfide bonds in the cytoplasm of these cells.

Replacement paragraph for page 50, at lines 27-32:

The open reading [fromes] frames coding for ahpCF were amplified from the chromosome of the wild type and the FA113 mutant by PCR and cloned into the pACYC derivative pLAC-YC. Constructs containing either the entire operon or just the *ahpC*-gene (wild type or mutant) were transformed into JL10 and DR456, in which in addition to the *trxB* and *gor* mutations, the *ahpCF* locus is also inactivated. DR456 is also referred to as “*trxB gor ahpCF::Km* mutant”. Growth of each of these strains was determined on rich medium (NZ).

Replacement paragraph for page 53, lines 18-24:

<u>Relevant genotype</u>	<u>Growth on rich medium</u>
TrxB gor	No
TrxB gor ahpC	Yes

TrxB gor ahpC* gshA	[Yes] <u>No</u>
TrxB gor ahpC* grxA	[Yes] <u>No</u>
TrxB gor ahpC* trxA trxC	<u>Yes</u>

Replacement paragraph for page 53, lines 25-26:

Example 12: Assay to determine whether AhpC* has glutathione (glutaredoxin) [] reductase activity

Copy of amended claims with changed marked thereon:

26. (Amended) The prokaryotic cell of claim 25, wherein the catalyst is a DsbC protein or an analog thereof which lack a signal peptide.
45. (Amended) The prokaryotic cell of claim 44, wherein the catalyst is a DsbC protein which lacks a signal peptide.

A triplet insertion in a *ahpC* restores growth to a *trxB* *gor* double mutant

wt
AhpC